*TWO L-ASPARAGINASES FROM E. COLI AND THEIR ACTION AGAINST TUMORS**

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Normal guinea pig serum inhibits the growth of a number of transplantable mouse and rat tumors *in vivo*, and evidence has been presented that this is brought about by its content of L-asparaginase.[1] Recently it has been shown that asparaginases from *E. coli*[2] and *S. marcescens*[3] also possess antitumor activity, but enzymes from other microbial sources have been ineffective.[2, 4] We have found that, depending upon the conditions of growth, *E. coli* cells possess one or two asparaginase activities. This is due to two distinct enzymes which differ in a number of properties, most significantly in their affinities for L-asparagine. The enzyme with the higher affinity markedly inhibited lymphomas in mice, while the asparaginase with the lower affinity was ineffective. Agouti serum, which contains an L-asparaginase with an affinity intermediate between those of the two enzymes from *E. coli*, inhibited tumors less than did the *E. coli* enzyme with the higher affinity. We conclude that the affinity of asparaginase for its substrate is related to its degree of effectiveness against sensitive tumors.

Materials and Methods.—*E. coli* K12 was grown with vigorous aeration in a 28-liter Fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) until the number of cells reached 10^8 per ml.[5] Growth was then either stopped by the addition of enough crushed ice to bring the temperature rapidly from 35 to 0° (continuously aerated culture) or allowed to proceed in the absence of aeration for 20 min at room temperature and then stopped by chilling (briefly anaerobic culture). Cells were harvested by centrifugation, broken in an X-Press (Biochemical Processes, Inc., Islip, N.Y.), and extracted with extract buffer (Tris-HCl buffer, pH 7.8, 10 mM; magnesium acetate, 10 mM; KCl, 30 mM; 2-mercaptoethanol, 5 mM) following our modification[5] of the procedure of Nirenberg and Matthaei.[6] The extracts were centrifuged at 105,000 × *g* for 2 hr; only the supernatant fraction was used.

Fractionation was performed at 0° by slow addition of solid ammonium sulfate to the extracts. Precipitates were dialyzed against extract buffer. Chromatography on a column (0.9 cm × 45 cm) of DEAE-cellulose (Serva Entwicklungslabor, Heidelberg, Germany) was carried out at 4°; samples were applied in 0.05 *M* NaCl in 0.01 *M* Tris-HCl buffer, pH 7.4, and eluted in a single step with 0.1 *M* NaCl at the same pH.

Serum was obtained by cardiac puncture of mature agoutis; this material is a rich source of L-asparaginase.[7]

Asparaginase activity was assayed by following the formation of either ammonia or aspartate. Incubations were carried out at 35°.

Formation of ammonia: Incubation mixtures contained 50 mM Tris-HCl buffer, pH 8, 10 mM magnesium acetate, and 5 mM 2-mercaptoethanol. Ammonia was determined after microdiffusion[8] by reaction with ninhydrin.[9]

Formation of aspartate: Incubation mixtures contained 50 mM Tris-HCl buffer, pH 7.8, 10 mM magnesium acetate, 30 mM KCl, and 5 mM 2-mercaptoethanol. Formation of radioactive aspartate from uniformly labeled C^{14}-L-asparagine (Nuclear-Chicago Corp., Des Plaines, Illinois) was measured after application of 10-μl samples of the reaction mixture to a strip (2 cm × 10 cm) of the strongly basic ion-exchange paper SB-2 (Reeve Angel Co., Clifton, N.J.) previously converted to the acetate form, and elution downwards with 0.5 *M* acetic acid. Aspartate was well separated from asparagine, and the section of the strip bearing aspartate was cut out and mounted on a metal planchet. Radioactivity was measured directly on the paper with a thin-window

Geiger counter (Nuclear-Chicago Corp.). With this technique it was possible conveniently to assay enzymatic activity with substrate at concentrations between 0.01 and 10 mM.

Results.—We obtained two extracts of *E. coli*, one from cells grown with continuous aeration, and the other from cells kept unaerated for a short period before harvesting. Both extracts catalyzed the formation of ammonia from L-asparagine at a concentration of 10 mM. When the concentration of asparagine was lowered to 0.1 mM, an additional asparaginase activity was revealed in extracts from cells kept briefly unaerated. Because of the great difference in their affinities for asparagine, the activities could be distinguished from each other even when both were present in the same extract. These results are presented in Table 1.

The assay at 0.1 mM asparagine appears to measure a distinct asparaginase of high avidity, while formation of ammonia at the higher concentration of substrate measures the sum of the two activities. The fraction of ammonia production due to enzyme with low affinity may be estimated by subtraction. Apparently its amount in *E. coli* cells was unaffected by the different conditions of growth and harvesting.

The two activities were also shown to be due to distinct enzymes because of different sensitivities to thermal inactivation. After 10 min at 55°, 87 per cent of the low-affinity activity was lost. Under the same conditions there was a decrease of only 36 per cent in the activity of the enzyme of high avidity. The two enzymes were separated by salt fractionation. Low-affinity enzyme was precipitated completely by ammonium sulfate at 55 per cent saturation, while 78 per cent of the high-avidity enzyme was recovered in the supernatant.

The activities of asparaginase in agouti serum and the two enzymes from *E. coli* were compared at different concentrations of substrate. It can be seen (Table 2) that enzyme from cells kept briefly unaerated has the greatest affinity for asparagine, the enzyme from agouti serum has rather less, and that from continuously aerated cells has least of all.

All three enzymes have also been examined by means of the sensitive assay technique described in earlier publications,[1] for their activity against Lymphoma 6C3HED implanted in C3H mice, except that injections of asparaginase were given 24 hr after implantation of tumor cells, instead of 1–2 hr as commonly practiced for

TABLE 1

ASPARAGINASE ACTIVITIES IN EXTRACTS OF *E. coli*

Cultural condition	Concentration of L-asparagine (mM)	Specific activity
Continuously aerated	10	270
	0.1	6
Briefly anaerobic	10	350
	0.1	96

Specific activities for measurements at 10 mM asparagine are given in mμmoles ammonia produced per min per mg extract protein; those at 0.1 mM are given in mμmoles aspartate produced per min per mg protein.

TABLE 2

COMPARISON OF ASPARAGINASE ACTIVITIES IN AGOUTI SERUM AND *E. coli* EXTRACTS AT VARIOUS SUBSTRATE CONCENTRATIONS

Source of enzyme	Concentration of L-asparagine (mM)	Relative velocity
Agouti serum, whole	0.05	25
	0.1	82
E. coli, continuously aerated, unfractionated extract	0.1	5
E. coli, briefly anaerobic extract purified as in text (*Materials and Methods*)	0.05	77
	0.1	100

Relative velocity is the ratio of the initial rate obtained at the indicated concentration of substrate to the rate measured at a saturating concentration. For enzyme from continuously aerated cells, this concentration of asparagine was 100 mM; for the enzyme from cells kept anaerobically, 0.1 mM; for agouti serum, 1 mM.

TABLE 3

EFFECT OF ASPARAGINASES FROM AGOUTI SERUM AND *E. coli* ON THE GROWTH OF LYMPHOMA 6C3HED

Treatment	Number of tumors implanted	Number of tumors completely suppressed	Day of tumor appearance	Standard deviation (days)
No enzyme	26	0	9	±1
Agouti serum (0.2 ml) 150 units	6	0	13	±1
E. coli high- + low-affinity enzymes (0.25 ml) 96 units	8	3	16	±2
E. coli low-affinity enzyme (0.45 ml × 2) 168 units	6	0	9	±0

Asparaginase activity was assayed by ammonia production at a concentration of asparagine of 10 mM. Units of enzyme: μmoles of ammonia formed per hour. Suspensions containing 50,000 Lymphoma 6C3HED cells were injected subcutaneously into C3H mice on day zero. Enzyme was given intraperitoneally on day 1 or days 1 and 2 to provide the total number of units shown.

serum enzymes. *E. coli* enzyme was ineffective at the earlier time.[10] In Table 3 it is shown that the high-avidity enzyme from *E. coli* produced complete suppression of three of eight implanted tumors when given in a single dose containing 96 enzyme units. By contrast, twice this dose of the low-affinity enzyme produced no effect whatsoever. The agouti serum occupied an intermediate position, producing a delay of four days in the appearance of tumors when 150 enzyme units were given, but not complete suppression of any tumor.

Discussion.—Undoubtedly a number of factors influence the effectiveness of asparaginases in inhibiting tumor growth. The asparaginase of yeast, which was found to be inactive against tumors, was cleared from the blood much more rapidly than the guinea pig serum enzyme.[4] Different rates of clearance of the three enzymes studied here cannot, however, account for the difference found in their activities against the lymphoma. The *E. coli* asparaginase of low affinity was cleared rapidly; its half life after intravenous injection was 15 min. Although the asparaginase with the high avidity was removed from the blood more slowly (a half life of 55 min), it was cleared considerably faster than the agouti serum enzyme. The latter enzyme had a half life of 11.3 hr and a much lower efficiency in inhibiting tumor growth.

Our results suggest that asparaginase acts by lowering critically the concentration of asparagine so that it becomes unavailable to a competing process essential for tumor growth. We assume that the two enzymes active against the lymphoma are distributed in a similar manner in treated mice and that the properties measured *in vitro* correspond to those *in vivo*. With these assumptions in mind we estimate that the concentration of asparagine is about 0.1 mM at the site at which tumor inhibition occurs. At this concentration the high-avidity asparaginase of *E. coli* is still saturated, whereas the enzyme in agouti serum is not, as shown in Table 2. If the concentration of asparagine were very much lower than 0.1 mM, neither asparaginase would have been effective, since both enzymes were found to be unsaturated at 0.05 mM.

These observations are consistent with direct measurements of the asparagine content of lymphoma cells, which were found to contain 0.13–0.14 mmoles of free asparagine per kg wet weight, and only 0.02–0.03 mmoles during treatment with asparaginase.[11] The *E. coli* asparaginase of low affinity is virtually inactive at these asparagine concentrations; this could account for its ineffectiveness against tumors. Its action might also be limited by its rapid removal from the blood.

Incubation *in vitro* of asparaginase-sensitive tumor cells with the enzyme from guinea pig serum resulted in the abrupt cessation of the incorporation of amino acids into protein.[13] Furthermore, one of us has shown that with cell-free extracts of *E. coli* containing high-avidity asparaginase, synthesis of protein under the direction of the RNA isolated from f2 bacteriophage was limited by the concentration of one single amino acid, asparagine.[5] Direct dependence of the rate of amino acid incorporation on the concentration of asparagine was observed up to 0.1 mM. Addition of asparagine failed to stimulate protein synthesis in extracts of *E. coli* containing only the low-affinity enzyme. It is likely that the essential role of asparagine in tumor growth is in the synthesis of protein. While no data are available for asparagine-activating enzymes, the concentrations of amino acids saturating other enzymes are of the same order of magnitude as that of asparagine needed to saturate the tumor-inhibiting asparaginases.[12] The possibility that the action of asparaginase on tumors may be comparable to that on protein synthesis in extracts of *E. coli* is at present under investigation.

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[1] Broome, J. D., *Nature*, **191**, 1114 (1961); *J. Exptl. Med.*, **118**, 99 (1963); *ibid.*, p. 121.

[2] Mashburn, L. T., and J. C. Wriston, *Arch. Biochem. Biophys.*, **105**, 450 (1964).

[3] Wriston, J. C., and J. D. Broome, unpublished observations.

[4] Broome, J. D., *J. Natl. Cancer Institute*, **35**, 967 (1965).

[5] Schwartz, J. H., these PROCEEDINGS, **53**, 1133 (1965).

[6] Nirenberg, M. W., and J. H. Matthaei, these PROCEEDINGS, **47**, 1588 (1961).

[7] Holmquist, N. D., *Proc. Soc. Exptl. Biol. Med.*, **113**, 444 (1963).

[8] Conway, E. J., *Microdiffusion Analysis and Volumetric Error* (London: Lockwood, 1947).

[9] Moore, S., and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

[10] This revealed a latent period between tumor cell implantation and the beginning of sensitivity to asparaginase. *E. coli* enzyme was removed from the blood rapidly, and none was detectable 24 hr after administration. By this time the concentration of asparagine in the blood had begun to rise. On the other hand, no latent period was seen with agouti serum. At 24 hr, substantial amounts of asparaginase remained in the blood, and at this time the concentration of asparagine in the blood had reached its lowest. Also, the asparaginase preparations inhibiting tumor growth at the early stage used in the present assay technique have proved very effective against larger, already palpable tumors.

[11] Broome, J. D., in *Proc. Ninth International Cancer Congress, Tokyo* (1966).

[12] Schweet, R. S., and E. H. Allen, *J. Biol. Chem.*, **233**, 1104 (1958); Bergmann, F. H., P. Berg, and M. Dieckmann, *J. Biol. Chem.*, **236**, 1935 (1961); Allende, C. C., and J. E. Allende, *J. Biol. Chem.*, **239**, 1102 (1964); Fangman, W. L., and F. C. Neidhardt, *J. Biol. Chem.*, **239**, 1839 (1964).

[13] Sobin, L. H., and J. G. Kidd, *J. Exptl. Med.*, **123**, 55 (1966).